Chapter 16

Melon (*Cucumis melo*)

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Abstract

Genetic transformation is an important technique used in plant breeding and to functionally characterize genes of interest. The earliest reports of *Agrobacterium*-mediated transformation in the melon (*Cucumis melo*) were from the early 1990s (Fang and Grumet, Plant Cell Rep, 9: 160–164, 1990; Dong et al., Nat Biotechnol 9: 858–863, 1991; Valles and Lasa, Plant Cell Rep 13: 145–148, 1994). These early studies described three problems that decreased the efficiency of transformation: tetraploidy, chimeras, and escape. Using a liquid culture system for somatic embryogenesis, Akasaka-Kenedy et al. (Plant Sci 166: 763–769, 2004) overcame these problems and established an efficient transformation system; the protocol introduced in this chapter is based on this method.

Key words *Agrobacterium*-mediated transformation, Chimera, Escape, Liquid culture, Somatic embryo, Tetraploidy

1 Introduction

In addition to being an important horticultural crop, the melon (*Cucumis melo* L.) is also a useful experimental organism for understanding fruit ripening, as physiological and biochemical changes in flavor development and texture occur during the ripening of the fruit [1]. An understanding of the molecular mechanism underlying melon fruit ripening will require the isolation and functional verification of genes contributing to this trait.

Transformation is a technique used to characterize the functions of genes of interest. In the last two decades, several types of genetic transformation techniques have been developed in the melon. *Agrobacterium*-mediated techniques reported in the early 1990s [2–4] identified three major problems affecting transformation in the melon: the induction of tetraploidy, chimeras, and escape. Embryogenesis via cotyledon explants was later found to reduce the occurrence of tetraploidy [5]. Embryogenesis is a useful regeneration system because the embryos originate from a single cell and the number of chimeric plants regenerated is therefore

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reduced. Problems with escape appear to be caused by the inefficient selection of transformed cells. Because the whole explant is exposed to antibiotics when suspended in liquid media but not when cultured on solid media, the liquid culture system achieves a more effective selection of transformed cells. Akasaka-Kenedy et al. [6] demonstrated that the use of a liquid culture system for *Agrobacterium*-mediated melon transformation reduced the occurrence of tetraploidy, chimeras, and escape. The protocol introduced in this chapter is based on the method of Akasaka-Kenedy et al. [6]. One mature melon seed was chopped into 12–20 segments for the preparation of explants for genetic transformation. These explants were cultured in liquid embryo induction (EI) medium containing MS salts, MS vitamins, 3 % sucrose, 2 mg/l 2,4-D, and 0.1 mg/l BA for 2 days. After this 2-day pre-culture period, the segments were inoculated with *Agrobacterium tumefaciens* containing the desired transgene and cocultured on solid EI medium with 0.8 % agar for 4 days in the dark. To select transformed calli and embryos, the inoculated segments were transferred to liquid EI medium containing 25 mg/l kanamycin and 375 mg/l Augmentin and subcultured every 2 weeks. Antibiotic-resistant embryos appeared on the surface of the explants 3–4 months after *A. tumefaciens* transfection. For regeneration and plant development, the embryos were cultured on solid MS medium containing 50 mg/l kanamycin and 375 mg/l Augmentin without plant growth regulators (PGRs) for 1 month. The transgenic melon plants were acclimated and grown in a greenhouse. Three transgenic lines were obtained from 130 explants that, in theory, could have been derived from 6.5 seeds. Thus, the transformation efficiency of this method was 2.3 % per explant and 46.1 % per seed.

2 Materials

2.1 Agrobacterium tumefaciens *Strain and Vector*

Agrobacterium tumefaciens GV2260 [7] carrying pIG121-Hm [8] was used in this protocol. This binary vector contains the neomycin phosphotransferase gene (*nptII*), beta-glucuronidase gene (GUS), and hygromycin phosphotransferase gene (*hpt*) cassette in T-DNA region. The expression of *nptII* gene is under control of Nos promoter, and the terminator is Nos terminator. Kanamycin is suitable for selection of transformed cells for melon transformation, so in this protocol, *nptII* gene and kanamycin were used for selection.

2.2 Plant Material

This protocol is optimized for *Cucumis melo* L. var *cantalupensis* 'Vedrantais' (Fig. 1). This cultivar was kindly provided by M. Pitrat Inra, Avignon, France, and it has been bred to homozygosity in laboratory and fields (*see* **Note 1**).

Fig. 1 *Cucumis melo* L. var. *cantalupensis* cv. 'Vedrantais'

2.3 Stock Solutions

2.3.1 MS salt

1. Stock 1 (50×): 82.5 g of NH_4NO_3, 95 g of KNO_3, 8.5 g of KH_2PO_4, 310 mg of H_3BO_3, 1,115 mg of $MnSO_4 \cdot H_2O$, 430 mg of $ZnSO_4 \cdot 7H_2O$, 41.5 mg of KI, 12.5 mg of $Na_2MoO_4 \cdot 2H_2O$, 1.25 mg of $CuSO_4 \cdot 5H_2O$, 1.25 mg of $CoCl_2 \cdot 6H_2O$, per 1,000 ml of distilled water. Store this solution at 4 °C.
2. Stock 2 (100×): 44 g of $CaCl_2 \cdot H_2O$ per 1,000 ml of distilled water. Store this solution at 4 °C.
3. Stock 3 (100×): 37 g of $MgSO_4 \cdot 7H_2O$ per 1,000 ml of distilled water. Store this solution at 4 °C.
4. Stock 4 (100×): 2.78 g of $FeSO_4 \cdot 7H_2O$ and 3.73 g of Na_2-EDTA per 1,000 ml of distilled water. Store this solution at 4 °C.

2.3.2 Vitamins and Phytohormones

1. MS Vitamin (200×): Dissolve 10 g of myo-inositol, 50 mg of nicotinic acid 50 mg of pyridoxine hydrochloride, 10 mg of thiamine hydrochloride, and 200 mg of glycine in 900 ml of distilled water and adjust the volume to 1,000 ml. Dispense the solution to 5 ml and store it at −30 °C.
2. 2,4-Dichlorophenozy acetic acid (2,4-D, 10 mg/ml): Dissolve 1 g of 2,4-D in 100 ml of dimethyl sulfoxide (DMSO). Dispense the solution to 1 ml and store it at −30 °C.
3. 6-Benzylaminopurine (BA, 1 mg/ml): Dissolve 0.1 g of BA in 100 ml of DMSO. Dispense the solution to 1 ml and store it at −30 °C.

2.3.3 Antibiotics and Selective Agents

1. Kanamycin (50 mg/ml): Dissolve 2 g of kanamycin in 40 ml of distilled water. And sterilize with a 0.22 μm cellulose acetate filter and store it at −30 °C.
2. Augmentin: Dissolve 1 tablet of Augmentin (GlaxoSmithKline K.K., Uxbridge, UK) in 5 ml of stilled water before using.

2.4 Culture Media

2.4.1 For Agrobacterium

1. LB medium (liquid): Dissolve 10 g of sodium chloride, 10 g of Bacto-tryptone, 5 g of yeast extract in 800 ml of distilled water. And adjust the volume to 1,000 ml with distilled water. The adjusted medium was autoclaved at 121 °C for 15 min. After autoclaved, it is cooled less than 60 °C and then antibiotics are added in LB medium.
2. LB medium (solid): 20 g of Bacto Agar were added to LB liquid medium before autoclaved. After autoclave, the medium was cooled at 60 °C and then antibiotics are added.

2.4.2 For Melon

1. Embryo induction (EI) medium: 30 g of sucrose was dissolved in 800 ml of distilled water, and add 20 ml of MS salt stock 1, 10 ml of MS salt stock 2, 10 ml of MS salt stock 3, 10 ml of MS salt stock 4, 5 ml of MS vitamin, 200 μl of 2,4-D (final concentration was 2 mg/l), and 100 μl of BA (final concentration was 0.1 mg/l) mixed well. After the sucrose was dissolved completely, pH was adjusted to 5.8 by sodium hydroxide, and then the volume was 1,000 ml total. The medium was autoclaved at 121 °C for 15 min. After autoclave, the medium was cooler than 60 °C; kanamycin and augmentin were added as necessary.
2. Cocultivation medium: 8 g of agar were added to EI medium just before autoclave.
3. Germination medium: 30 g of sucrose was dissolved in 800 ml of distilled water, and 20 ml of MS salt stock 1, 10 ml of MS salt stock 2, 10 ml of MS salt stock 3, 10 ml of MS salt stock 4, 5 ml of MS vitamin, and 8 g of agar were added to EI medium just before autoclave. The medium was autoclaved at 121 °C for 15 min. After autoclave, the medium was cooler than 60 °C; kanamycin and augmentin were added as necessary.

3 Methods

The various steps of the protocol are summarized in Fig. 2 and described in further detail below.

3.1 Growing Donor Plants in Green House

1. Seeds were wrapped with moisten paper towel for 1 week for germination.
2. Germinated seedlings were in soil in 10 cm pot with molding. The seedlings were grown in the greenhouse. The photoperiod and light intensity are natural condition in Tsukuba, Japan (36.0 N°, 140.0 E°). Lowest temperature is 15 °C, and highest temperature is 25 °C (*see* **Note 2**).
3. After the three leaves' appearance, transplant each plant into a 40 cm pot with molding (one plant per pot).

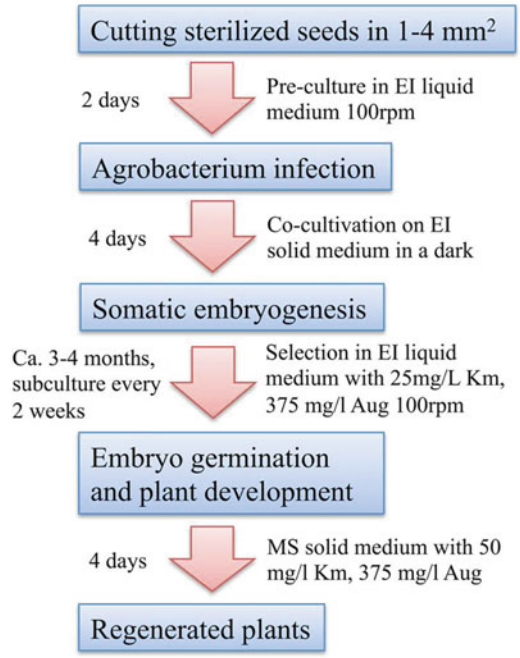

Fig. 2 Schematic of *Agrobacterium*-mediated transformation via somatic embryogenesis. The time schedule is indicated on the *left*. EI: MS medium containing 3 % sucrose, 2 mg/l 2,4-D, and 0.1 mg/l BA. Km and Aug indicate kanamycin and Augmentin, respectively

4. One week after transplant, melons were bending brunch by strings hanged by the beam of ceiling. Water was supplied, when the soil is dry.
5. Under ten nodes, axillary buds were cut off. Top pinched after 24 nodes appearance.
6. One month after planting, flowering was started. The female flowers were covered on the evening 1 day before flowering to avoid closing another plant.
7. In early morning (before 8:00 am), the female flowers were uncovered. Removing the petals on the flower, the flower was closed with the male flower on the same plant. If a few melons were fruited on one plant, remove all fruit leaving the best one.
8. About 50 days after pollination, melon fruits were harvested and incubated on dark and cool place for 1 week. Then the seeds were harvested.
9. Seeds were incubated at room temperature, for a week to dry up completely. The seeds were stored at 4 °C until using.

3.2 Plant Preparation

1. The seed coats were removed.
2. Seeds were sterilized with 70 % EtOH for 10 s and with 0.02 % hypochlorite with 0.01 % Tween 20 for 15 min (Fig. 3a).
3. Seeds were rinsed three times in distilled water for 5 min.
4. The seeds were soaked in sterile distilled water for 6 h.
5. The sterilized seeds were cut in half lengthwise and sectioned crosswised, producing explants approximately 1–4 mm^2 in size (Fig. 3b). Twelve to twenty explants were prepared from each segment.
6. The segments were cultured for 2 days in 10 ml of liquid EI medium with 100 ml volume of Erlenmeyer flask on a rotary incubator shaker (100 rpm) at 25 °C under a 16 h photoperiod (50 μmol/m^2/s).

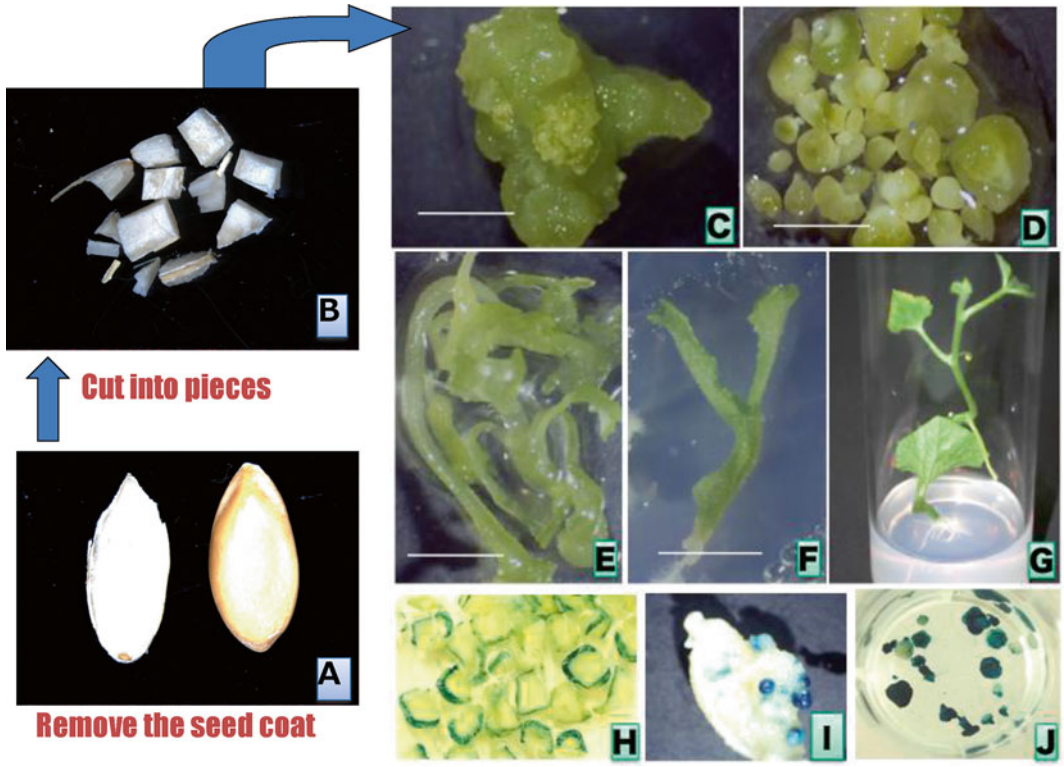

Fig. 3 Plant regeneration and transformation in the melon (*C. melo* L. var. *cantalupensis* cv. 'Vedrantais'). (**a**) Sterilized melon seeds without seed coats. (**b**) Sterilized seeds are cut into 1–4 mm^2 pieces. (**c**) Calli formed at the cut surfaces of explants after 3 weeks of culture (bar = 2 mm). (**d**) Embryos developed in liquid EI medium after 4 weeks of culture (bar = 4 mm). (**e**) Regenerated plants exhibited severe vitrification in liquid MS medium after 8 weeks of culture. (**f**) Healthy plantlets growing in 1.0 % agar-solidified MS medium after 8 weeks of culture (bar = 6 mm). (**g**) A regenerated plant obtained from a somatic embryo after 12 weeks of culture. (**h**) *Blue spots* were observed at the cut surfaces of explants 4 days after infection. (**i**) Strong GUS expression was observed on proliferated calli on the explants grown in liquid EI selection medium 6 weeks after infection. (**j**) Stable GUS expression was observed throughout the embryo 9 weeks after infection (color figure online)

3.3 Preparation of Agrobacterium Culture for Infection

1. *A. tumefaciens* was cultured on solid LB medium at 28 °C for 2 days.
2. A single colony was selected and cultured in 2 ml of LB medium at 28 °C and 200 rpm for 2 days until the culture reached the stationary phase.
3. A 15 μl volume of this culture was added to 15 ml of fresh LB medium; this mix was then cultured at 28 °C and 200 rpm for 20 h.
4. When the optical density of the culture reached 0.8–1.0, the cells were centrifuged and the pelleted bacterial cells were resuspended in 30 ml of liquid EI medium. The optical density (OD_{600}) was adjusted to 0.4–0.5.

3.4 Agrobacterium-Mediated Transformation

1. Melon explants pre-cultured for 2 days in liquid EI medium were inoculated with *A. tumefaciens* as described above and incubated for 20 min.
2. The inoculated explants were wiped with distilled filter paper to remove excess bacterial suspension.
3. Inoculated segments were cultured on EI medium with 0.8 % agar (Wako Pure Chemical Industries, Japan) for 4 days in the dark at 25 °C.
4. Melon explants inoculated with *A. tumefaciens* harboring pIG121-Hm showed GUS staining, indicating successful transformation (Fig. 3h–j).

3.5 Selection for Putative Transgenic Embryos and Calli

1. After cocultivation, the explants were cultured in 10 ml of liquid EI medium containing 25 mg/l kanamycin and 375 mg/l Augmentin with 100 ml volume of Erlenmeyer flask on a rotary incubator shaker (100 rpm) at 25 °C under a 16 h photoperiod with light intensity of 50 μmol/m^2/s (*see* **Note 3**).
2. The explants were subcultured and washed in water every 2 weeks (*see* **Note 4**).
3. After 3–4 months of culture, somatic embryos and calli were observed at the cut surfaces of the explants (Fig. 3c, d, i, and j).

3.6 Regeneration of Transgenic Plants

1. To induce embryo germination and plant development, the explants were transferred to 1.0 % agar-solidified germination medium containing 50 mg/l kanamycin and 375 mg/l Augmentin at 25 °C under a 16 h photoperiod (50 μmol/m^2/s) for 4 weeks (Fig. 3f and g) (*see* **Note 5**).
2. The transformation efficiency was approximately 2.3 %, with three transgenic plants obtained from 130 explants that, in theory, could have been derived from 6.5 seeds [6]. Escape was very low with this protocol: only 16.7 % of the kanamycin-resistant embryos were escapes. This protocol

also yielded an efficient regeneration of diploids in 'Vedrantais': approximately 75.9 % germinated from somatic embryos and greater than 60 % of the plants regenerated from the embryos were diploid.

3.7 Transplanting and Acclimation

1. Rooting shoots were checked polyploidy and select diploid plants.
2. Plants were planted in soil in 10 cm pots and covered over plastic filter for 1 week in culture room.
3. Make the tiny holes on the cover, for acclimation, and grow the plant for 1–2 weeks in culture room.
4. The pots were uncovered and plants were grown in a greenhouse as describe in Subheading 3.1.

4 Notes

1. Melon cultivars: The protocol described is applicable to other melon varieties such as *C. melo* L. var. *reticulatus* cv. 'Earl's Favourite'.
2. The suitable temperature for melon cultivation is between 15 and 25 °C. And melon grows well in low humidity condition.
3. Escape and chimeras: In this protocol, a liquid culture system was used to select transgenic embryos derived from explants. Because somatic embryos derive from a single cell and because whole embryos are exposed to kanamycin, the occurrence of escape and chimeras was reduced, even though a lower concentration of kanamycin was used than in the standard melon protocol. This result indicates that a liquid culture system is effective for the selection of transformed embryos in the melon.
4. Overgrowth of *Agrobacterium*: When using a liquid culture selection system, it is difficult to eliminate *A. tumefaciens* from liquid medium containing sugar during the selection of transgenic embryos. Particular care must therefore be taken to change the liquid EI medium when using this protocol. During the subculture, the explants must be washed in sterilized water and new culture vessels must be used.
5. Vitrification: Melon tissues are sensitive to vitrification. Somatic embryos derived from liquid culture have often been found to exhibit typical vitrification and fail to develop into plantlets [5]. To avoid vitrification, 1.0 % agar-solidified G medium was used for germination in this protocol. Embryos did not grow in 0.4 % Gelrite-solidified medium. Using surgical tape to seal, the culture vessels were also important for keeping the humidity low during plant regeneration.

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